Small-Diameter Nerve Fiber Neuropathy in Patients with Systemic Autoimmune Diseases

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Scientific environment

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Contents

Acknowledgements

Already as a medical student in Copenhagen I was introduced to clinical research. In 1988 I came to Stavanger, and professor Kenneth Dickstein and later on professor Dennis Nilsen and dr.med Harald Bergrem recruited me for clinical research.

In 1990 I came to the Department of Rheumatology at the University Hospital of Northern Norway where professor Roald Omdal was a research fellow. The same year I wrote my first scientific article in cooperation with him. We both moved back to Stavanger where professor Omdal continued his clinical research on systemic lupus erythrmatosus (SLE) in cooperation with professor Svein Ivar Mellgren at the Department of Neurology at the University Hospital of Northern Norway. They built up a methodology for visualizing intraepidermal nerve fibers as part of their research on patients with SLE.

In year 2000 professor Omdal recruited me to clinical research on small-diameter nerve fibers in immunological diseases. In 2004 I joined the PhD program at the Institute of Internal Medicine, University of Bergen, and I got financial support from the University Hospital's research funding (Helse Vest).

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Abbreviations

ACR	American College of Rheumatology
ANOVA	Analysis of variance
SSA/Ro	Sjögren's syndrome A antigen
SSB/La	Sjögren's syndrome B antigen
ARA	American Rheumatism Association
DAS 28	Disease Acitivity Score 28
dsDNA	Double stranded DNA
ENF	Epidermal nerve fibers
Ig	Immunoglobulin
i.e.	Id est
IENF	Intraepidermal nerve fibers
MHAQ	Modified Health Assessment Questionnaire
NCS	Nerve conduction studies
NGS	Normal gout serum
NIS	Neuropathy Impairment Score
NSC	Neuropathy Symptom and Change Score
PBS	Phosphate-buffered saline
PGP 9.5	Protein gene product 9.5
PN	Peripheral neuropathy
PSS	Primary Sjögren´s syndrome
RA	Rheumatoid arthritis
SD	Standard Deviation
SLE	Systemic lupus erythematosus
SLEDAI	SLE Disease Activity Index
SLICC	Systemic lupus international collaborating clinics
TBS	Tris-HCl buffer solution

List of publications

I. Omdal R, Mellgren SI, Gøransson L, Skjelsol A, Lindal S, Koldingsnes W, Husby G. Small nerve fiber involvement in systemic lupus erythematosus: a controlled study. Arthritis Rheum 2002;46:1228-32.

II. Gøransson LG, Mellgren SI, Lindal S, Omdal R. The effect of age and gender on epidermal nerve fiber density. Neurology 2004;62:774-7.

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Introduction

Peripheral neuropathy (PN)

Peripheral neuropathy is a common neurologic disorder of diverse etiologies. Establishing the diagnosis may sometimes be difficult, and the highest likelihood predicting PN is the combination of multiple symptoms, multiple signs, and abnormal nerve conduction studies (NCS) (1). The patient's symptoms and signs differ depending on which part of the peripheral nervous system is involved. In pure *motor* PN muscle weakness without sensory loss is the primary finding, and in pure *sensory* PN symptoms like numbness, tingling, or pain are prevailing. Upon neurologic examination evidence of both sensory and motor involvement are often revealed. In addition the patients may have symptoms and signs from the autonomic nervous system like orthostatic fall in blood pressure, heat intolerance, or bowel-, bladder-, or sexual dysfunction. No neuropathic symptoms, findings or tests are, however, consistently abnormal (2), and different composite scores with combinations of clinical findings and test abnormalities have been proposed for establishing a diagnosis (1,2).

In PN all types of nerve fibers are usually involved, and the function of the fast conducting large-diameter nerve fibers can be evaluated through conventional neurologic examination by testing deep tendon reflexes and muscle strength. NCS, however, give a more accurate and objective measure of large-diameter nerve fiber function.

In some patients selective pathological processes involving only small-diameter nerve fibers (myelinated Aδ- and unmyelinated C-fibers) sometimes occur (3,4). Small-fiber neuropathy is classified as a subtype of PN in which both sensory and autonomic nerve fibers may be involved (5,6). The patients typically have positive sensory symptoms like tingling, burning, prickling or shooting pain (5,6). Negative sensory symptoms like decreased or abolished ability to perceive cold, warmth, or noxious stimuli are less likely to be reported (7), and pure autonomic neuropathies are rare (6). Small-fiber neuropathy is reported in various diseases like diabetes mellitus, HIV infection, Fabry disease, alcohol abuse, and systemic amyloidosis, although most cases are idiopathic (3,5,6,8-10). These patients will have no neurologic deficits except impairment of pain and temperature sensation and/or autonomic disturbances, and the nerve conduction velocities will be normal unless larger nerve fibers are involved.

The small-diameter myelinated Aδ- and unmyelinated C-fibers functions are most commonly investigated by quantitative sensory testing devices for determination of perception thresholds to warmth, cold, and pain (6). In addition, other tests like sympathetic

skin response, quantitative sudomotor axon reflex, microneurography, somatosensory-evoked cortical potentials, laser-evoked potentials, contact heat-evoked potentials, cutaneous silent period, and cardiovagal and adrenergic autonomic tests have been or are used in the investigation of small-fiber neuropathy (5,6). However, none of the tests are established as standard methods for testing small-diameter nerve fiber function.

Until recently, no easily feasible test to evaluate the density of small-diameter nerve fibers existed. By immunostaining the neuropeptide protein gene product 9.5 (PGP 9.5) it became possible to visualize small-diameter nerve fibers by immunohistochemical techniques (11). Since the small-diameter nerve fibers traverse the skin perpendicular to the epidermis, the density of small-diameter intra-epidermal nerve fibers (IENF) can be quantitated in punch skin biopsies, and normative reference ranges for densities of these nerve fibers are available (12). This method has later been considered an objective and reproducible procedure for evaluation of small-diameter nerve fiber densities (12,13).

Systemic lupus erythematosus (SLE)

SLE is a chronic inflammatory autoimmune disease. It is characterized by a variety of clinical and immunological abnormalities, and the principal underlying immune disorder is an abnormal cellular and humoral immune response with excessive T-cell help, polyclonal B-cell activation, and the production of numerous auto-antibodies of diverse specificities (14). The autoantibodies may be directed against components of the cell nucleus of which antibodies against nucleosomes, double-stranded DNA (dsDNA) and histones are believed to be of pathogenetic significance (15-17). The type I interferon system has been proposed to have a central role in the pathogenesis of SLE, and it has been shown that necrotic and late apoptotic cells release material that in combination with IgG from SLE patients can induce production of interferon α in plasmacytoid dendritic cells generating autoimmune T and B cells (18-20).

The clinical spectrum of the disease is wide, from an almost asymptomatic clinical presentation to a severe life-threatening disease affecting several internal organs. The prevalence varies in different reports from 36-68 cases per 100.000 persons with an incidence of approximately 3-5 per 100.000 persons (21-23). A recent Scandinavian study reports better survival, less renal involvement and therefore a higher prevalence of the disease, and the median age at diagnosis in this study was 47 years (23). This may be due to a higher awareness among physicians and/or more aggressive treatment with better survival. The five and 10 year survival rates were 93% and 83% respectively in a recent Swedish study, and only the 10 year survival was reduced compared to age and sex matched healthy individuals

(23). However, the mortality rate is about threefold higher in Blacks with SLE, and twofold higher in Asians compared to Caucasians (24). The incidence and prevalence of SLE is higher in women compared to men, and there is also a higher incidence in African-Americans compared to whites (3,25). The female to male ratio at onset before puberty is 2.0, peaks to 8.0 in the forties and then declines to 2.3 after 60 years of age (26). These observations point to genetic, environmental, and hormonal factors as important players for the development of SLE.

The diagnosis of SLE is based on symptoms and clinical findings as well as on hematologic and biochemical abnormalities like detection of nuclear autoantibodies, anti-phospolipid antibodies, or positive anti-phospholipid antibody dependent tests. The American Rheumatism Association (ARA), later the American College of Rheumatology (ACR), first published preliminary classification criteria for SLE in 1971 and revised them in 1982 and 1997, when a positive LE cell preparation was replaced with abnormal serum levels of antiphospholipid antibodies and/or a positive test result for lupus anticoagulant (Table 1) (27,28). A patient is classified as having SLE if any four or more of the 11 criteria are present serially or simultaneously during any interval of observations. The classification criteria were developed primarily for research purposes, but are also useful for evaluating individual patients.

There has not been a universal agreement on evaluation of the disease activity in patients with SLE, and about 60 different indices have been proposed (29). A disease activity index is important for evaluating the response to treatment and in research for comparison between different groups of patients with SLE. The systemic lupus erythematosus disease activity index (SLEDAI) is a model based on clinicians' global judgements of various clinical variables for describing activity in patients with SLE (30). SLEDAI is a validated instrument based on 24 variables from nine organ systems (30). The maximum score is 105, but few patients will have scores greater than 45. A flare or improvement of disease activity is proposed as an increase or decrease in SLEDAI of > 3, and complete remission as a SLEDAI of 0 (31). Organ damage caused by the disease is evaluated by a damage index defined by the systemic lupus international collaborating clinics (SLICC), as an irreversible change in an organ or system that has occurred since the onset of SLE and is present for at least six months (32). Twelve organ systems are scored, and the SLICC damage index increases with disease duration and is associated with mortality (33).

PN in SLE is reported to occur with a prevalence of 5 – 27% dependent on criteria applied and patient population studied, and is characterized by a length dependent mild

sensory or sensorimotor neuropathy (34-37). Usually, the neuropathic process is modestly progressive over time, but may fluctuate and is not necessarily irreversible (38). Moreover, mono-neuropathies or multiple mono-neuropathies can sometimes occur on the basis of a vasculitic process or as part of a more generalized neuropathic process in the nervous system.

Primary Sjögren´s syndrome (PSS)

PSS is an autoimmune disease mainly affecting exocrine glands and is clinically characterized by symptomatic dryness of mucous membranes including the eyes (keratoconjuntivitis sicca) and oral cavity (xerostomia) (39). The histological hallmark is a focal infiltration of mononuclear lymphoid cells, predominantly T-cells with fewer B-cells and macrophages, in the exocrine glands replacing glandular epithelium (39). A large number of autoantibodies have been reported of which the non-organ specific autoantibodies anti-SSA (Ro) and anti-SSB (La) are best characterized. Onset of the disease is usually insidious, and more than half of the patients develop extraglandular manifestations like myalgias, arthralgias, and involvement of the pulmonary-, and gastrointestinal system (40). General and unspecific phenomena like fatigue are also considered frequent. PSS is a worldwide disease and may occur in all ages with a peak incidence in the fourth and fifth decade of life and a female to male ratio of 9:1 (40). The prevalence is estimated not to exceed 0.6% (40,41).

The diagnosis of PSS is, like for SLE, in clinical practice often based on a pragmatic use of symptoms, signs, laboratory, and histological features. For research purposes classification criteria have been published, but until recently several sets of diagnostic criteria existed resulting in non-comparable findings in different studies (42-45). In 2002 an international consensus group published revised classification criteria for primary- and secondary Sjögren's syndrome (46). Having PSS according to this classification requires four of six criteria including positive lip biopsy and/or positive antibodies to anti-SSA and/or anti-SSB (Table 2) (46). The diagnosis can also be achived if three of the four objective criteria are present. No validated disease activity index exists for PSS.

The most common abnormality in the peripheral nerves in patients with PSS has reportedly been a symmetric sensorimotor PN followed by a symmetric pure sensory PN (47,48). However, to our knowledge no longitudinal or cross sectional studies have been published in support of this. Also, the reported prevalence of neurologic manifestations vary between published studies most likely due to selection bias and not at least because of the changing diagnostic criteria for PSS during the last few years.

In PSS a T-lymphocyte infiltration in the dorsal root ganglia affecting sensory neurons have been reported as part of a ganglionitis (49). This condition is probably an infrequent phenomenon and is clinically characterized by gait and limb ataxia and also hyperalgesic symptoms at proximal sites. The symptoms in these patients do not follow the typical dying-back process of axonal neuropathies with symptoms starting in the distal part of the limbs but involve both proximal and distal areas of the body. In healthy subjects the IENF densities are reported to be higher in the proximal thigh compared to the distal leg generating a proximal-distal gradient (12). However, in patients with ganglionopathies no such gradients are reported (50). In these patients the inflammatory process in the ganglia results in degeneration of the sensory neurons, which is reflected in a non-length distribution of symptoms, abnormal NCS in both upper and lower limbs, and also by reduced densities of IENF in both proximal and distal sites of the limbs resulting in no proximal-distal gradients in IENF densities (50).

Rheumatoid arthritis (RA)

RA is a chronic, inflammatory, symmetrical, destructive arthritis primarily involving the metacarpophalangeal-, proximal interphalangeal-, wrist- and metatarsophalangeal joints. Extra-articular manifestations usually reflect severe disease often accompanied with high levels of rheumatoid factor and signs of high inflammatory activity (51). The prevalence in most populations is 0.5 – 1.0% (52-54). The incidence is, however, declining, and the reported incidence is today 25-50/100,000 with a 4:1 ratio of women to men in the 35-44 age group compared to 1:1 in the 75-84 age group (54-56). The declining incidence might implicate specific environmental factors among which temporal changes in the incidence of infectious agents and exposure to the contraceptive pill have been proposed (55,57).

Multiple cells contribute to the chronic inflammation, but synovial fibroblasts seem to be the key cells in the propagation of RA (58). The synovial fibroblasts secrete a number of proangiogenic factors and proinflammatory factors that activate T-cells inducing T-cell proliferation and production of cytokines (58,59). The inflammatory process stimulates pannus formation, bone-, and cartilage damage (58,59).

Compression neuropathies have been considered common in RA (60), although a controlled study disputed this by disclosing more carpal tunnel syndromes in healthy subjects compared with patients with RA (61). A mild distal symmetric sensory neuropathy is considered a late complication to the disease. Rarely, mono- and multiple mono-neuropathies are reported in association with the very serious condition of rheumatoid vasculitis (60).

Aims of the study

1) To establish normative values for IENF densities based on findings in healthy subjects and to test the intra- and interobserver variation for the method.
2) To investigate the prevalence of small-diameter nerve fiber neuropathy in patients with chronic inflammatory diseases with main focus on SLE.
3) To investigate if small-diameter nerve fiber neuropathy is an isolated phenomenon in patients with SLE and PSS or if it is part of a more generalized and diffuse neuropathy involving both small- and large-diameter nerve fibers.
4) To investigate the spectrum of peripheral neuropathy in patients with PSS fulfilling the revised international diagnostic criteria.
5) To investigate if the involvement of small-diameter nerve fibers varies between different chronic inflammatory diseases like SLE, PSS and RA.

Subjects and methods

Patients and healthy subjects

The patients in these studies were recruited in the following manner: In paper I the patients were recruited from the outpatient clinic of the Department of Rheumatology, University Hospital of North Norway, Tromsø. In paper III – V from the Department of Internal Medicine, Stavanger University Hospital. In paper V the patients with RA were recruited from the outpatient clinic of the Department of Rheumatology, Haukeland University Hospital. The University Hospital of North Norway provides local hospital services to about 150,000 inhabitants of Troms county, and central and regional hospital services to the counties of Troms, Finnmark and part of Nordland, with at total of 450,000 inhabitants. Most SLE patients are treated on a local hospital basis. Stavanger University Hospital provides local hospital services to about 290,000 inhabitants of Rogaland county, and Haukeland University Hospital to about 370,000 inhabitants of Hordaland county. In the southern part of Rogaland county (290,000 inhabitants) all patients with systemic autoimmune diseases are allocated to Stavanger University Hospital, and the studies on SLE and PSS originating from this area may therefore be considered close to population based.

In paper I, 15 consecutive and non-selected patients with SLE fulfilling the revised ACR criteria for SLE (28) were recruited. The age- and sex matched controls were friends and neighbors of the hospital staff.

In paper III – V, all medical records of in- and outpatients at Stavanger University Hospital with a diagnosis of SLE from 1980 through 2003 were reviewed. We identified 73 patients with SLE, all Caucasian, fulfilling the revised ACR criteria for SLE (28), and 60 patients gave their consent to participate. For PSS all medical records from 1980 through 2004 were reviewed, and in addition descriptions of positive lip biopsies diagnosed in the hospital's Department of Pathology were retrieved and the patients were thereafter screened for PSS. We identified 67 patients with PSS fulfilling the new revised international diagnostic criteria for PSS (46), and 62 patients gave their consent to participate. In addition an unselected group of 52 consecutive patients with RA fulfilling the ACR criteria (62) were recruited. All patients were reexamined whenever in doubt for fulfilling the diagnostic criteria, and only patients and healthy subjects giving informed consent were included.

The 106 healthy subjects in paper II were recruited from friends, hospital staff, and friends of the former. Healthy subjects were asked about their use of drugs and screened for concomitant conditions possibly associated with PN such as diabetes mellitus, thyroid disease,

and alcohol abuse.

Clinical examination

All patients with SLE or PSS were subjected to a standardized clinical and neurological examination by an experienced internist and a neurologist, respectively, and the patients were classified as having neuropathy or not according to the neurologist's standards of conventional neurologic examination. In Tromsø and also in Bergen, only one experienced rheumatologist performed the general clinical examination.

Quantitative scoring systems for neurological symptoms and signs

Quantitative scoring systems were used to score neuropathic symptoms and signs and in combination with other tests like NCS to judge the presence and severity of neuropathy in any given patient (63).

Symptoms. Neuropathy Symptom and Change score (NSC) is a standardized questionnaire for neuropathic symptoms based on an interview by a neurologist (2,7,64). The NSC scores are derived from answers to 38 questions (muscle weakness, sensation and autonomic symptoms). Number equals number of symptoms and the severity equals number of symptoms multiplied by severity (1 = mild, 2 = moderate, and 3 = severe). The questions are also subdivided by anatomical site, by positive and negative sensory symptoms, and by large-diameter and small-diameter nerve fiber sensory symptoms. In the SLE study only items for sensory symptoms were recorded according to the hypothesis of involvement of small-diameter nerve fibers as the main interest. Subjects without neuropathy will typically have a score of 0.

Signs. Neuropathy Impairment Score (NIS) is a graded evaluation of neuropathic impairment obtained by a standardized neurologic examination and scored into four subsets: cranial nerve function, muscle weakness, reflexes and sensory deficits (2,63,64). Age, sex and physical fitness are also taken into account by the examiner. Typically, persons without neuropathy will have a score of 0, and a score $\geq$ 2 is regarded as abnormal (2).

Nerve conduction studies (NCS)

Patients with SLE or PSS were examined. Surface stimulating and recording electrodes under standard temperature conditions were used and the results were corrected for patients' height. The amplitude, velocity, distal latency and the F-response of motor fibers of the median,

ulnar, peroneal, and tibial nerves were recorded bilaterally, as well as the amplitude, and the velocity of sensory fibers of the median, ulnar and sural nerves. In Stavanger we recruited 82 healthy subjects for establishing normative values for sensory data (mean ± 1.96 SD). For motor NCS the manufacturer´s (Dantec Keypoint apparatus, Dantec Medical A/S, Skovlunde, Denmark) recommendations based on data from the Department of Clinical Neurophysiology, University Hospital, Uppsala, Sweden, were used. Abnormal NCS in two or more nerves were defined as the neurophysiological criterion for PN (65). At the University Hospital of Northern Norway, Tromsø (Paper I) the neurophysiological laboratory's reference values were used for both sensory and motor nerves.

Skin punch biopsies

The skin biopsies were performed with a 3 mm disposable circular punch needle (Biopsy Punch, Stiefel Laboratories Ltd., Sligo Ireland) with a sterile technique after local anesthetizing with 2% lidocaine with adrenaline. Two biopsies were obtained from each person on the same leg in the same procedure approximately 10 cm above the lateral malleolus. The biopsies were taken from the right leg. The left leg was used if the skin on the right leg was inflamed or had scars. In patients with PSS and in a group of healthy subjects two biopsies were also obtained from the proximo-lateral part of the thigh about 20 cm distally from spina ilica anterior with the same technique as from the leg.

The specimens were immediately fixed in 2% paraformaldehyde, lysine and periodate fixative at + 4°C. After 12-24 hours, the biopsies were rinsed in 0.1M Sørensen`s buffer and placed in cryoprotectant solution of 20% glycerol/ Sørensen`s buffer at + 4°C overnight. The next day 50 µm sections were produced in a Leica CM 3050 cryostat. Alternatively, mounted specimens were frozen in liquid nitrogen and stored in - 70°C freezer for later sectioning. After cutting, the sections were stored in antifreeze solutions (mixture of glycerol (30%) and ethylene glycol (30%) in Sørensen`s buffer) for up to one week at - 20°C before used for immunostaining.

Sections were rinsed twice, 10 minutes each time, in 0.25M TRIS-HCL buffer (TBS)/1.5% NaCl, pH 7.4 in microtiter wells. The sections were transferred into 0.25% potassium permanganate for 15 minutes, and subsequently rinsed for 10 minutes in TBS. Bleaching in 5.0% oxalic acid for 5 minutes was followed by two rinses in TBS.

For protein blocking, sections were then transferred into 1.0% Triton-X 100 and 4% normal goat serum (NGS) (Dako, Denmark) in 0.25 TBS with 0.5% milk powder for 4 hours

at room temperature on a shaker table. The sections were then incubated at + 4°C overnight in wells containing 0.1% rabbit polyclonal antibodies to human PGP 9.5 (Chemicon International, Temecula, CA, USA) suspended in 0.5% Triton-X and 2% NGS in TBS with 0.5% milk. Subsequently the sections were rinsed in TBS and incubated for 1h in 1% secondary antibody (goat anti-rabbit IgG, Dako, Denmark) in 0.5% Triton-X 100/2% NGS in 0.25M TBS. Afterwards, the sections were rinsed in 0.25M TBS and endogenous peroxidase blocked with 1% H_2O_2 in 40% methanol/phosphate buffered saline (PBS), and then rinsed in 0.01M PBS. They were subsequently placed in avidin-biotin complex solution (1% streptavidin (Dako) in 0.01M PBS, and 1% biotinylated peroxidase (Dako)) for 1 hour at room temperature followed by rinsing in PBS. Thereafter the sections were transferred into Vector SG peroxidase substrate (Vector SG substrate kit for perocidase, Vector Labs, Burlingame, USA) and incubated for 2-10 minutes for the chromogen development reaction and rinsed again in PBS before mounting on slides in 0.01% Triton-X 100 in 0.01M PBS. After drying, the sections were counterstained with eosin and coverslipped with Histokit (Assistant, Sondheim, Germany).

The number of separate IENF in at least three sections from each biopsy was counted and the total length of epidermis was measured using the NIH Image 1.61 morphometry program (http:/rsb.info.nih.gov/nih-image/). The number of nerve fibers per mm was then reported as the mean of counts in six sections, three from each of the two biopsies. Only the single fibers and not the branches from the same IENF were counted. One single observer assessed all the biopsies, except in the interobserver part of the study (Paper II).

Blood and urinary samples

Healthy subjects were screened for cobalamin deficiency, and thyroid disease. Patients with SLE, PSS, and RA were screened with hematological and biochemical tests (plasma glucose, cobalamin, folic acid, and thyroid function tests), antinuclear antibodies (ANA), and the complement factors C3 and C4. In addition anti-dsDNA antibodies, anti-SSA - and anti-SSB antibodies were tested for in patients with SLE or PSS respectively. Conventional urinary analyses with dip-stick and microscopy were also performed.

Disease activity instruments

In patients with SLE, the disease activity was evaluated using the SLEDAI (30). The theoretically possible range is 0 – 105, but most patients have an index of less than 15 and very few patients an index greater than 45. The index is generated from disease activity

manifestations in nine different organ systems as well as presence or absence of selected immune parameters. DAS28 was used in patients with RA as disease activity index (66) and a score > 5.1 was defined as high disease activity (66). No validated disease activity index exists in PSS.

Statistics

Variables normally distributed were subjected to parametric statistics. Simple or multiple regression analysis were used to test associations between normally distributed quantitative variables. Unpaired t-tests (2-tailed) or analysis of variance (ANOVA) were applied when testing for two or more groups of quantitative data. To allow for multiple testing, the Bonferroni corrected method was applied for three or more numbers of comparisons. Non-parametric statistics were applied for variables not normally distributed. The Spearman rank correlation test was used to test associations between such data. When appropriate, results are reported as mean $\pm$ SD as well as median and range.

To assess the reliability of IENF counting in skin punch biopsies, we estimated the intra- and interobserver agreements. The intra- and interobserver variability was estimated by calculating the arithmetric mean and the absolute differences between repeated measurements of the same biopsy. Agreement plots according to Bland and Altman were constructed (67). By this method any systematic differences between observers, or by intraobserver readings of IENF will result in the mean of the differences being significantly different from zero. The wider the scatter between the points in the direction of the Y-axis, the worse will be the agreement. We will expect 95% of the difference between paired counting's to lie within $\pm$ 2 SD or more precisely $\pm$ 1.96 SD of the mean, which is defined as the limit of agreement (67). Providing the differences are acceptable in clinical practice, the intra- and interobserver agreements are accepted.

The statistical analysis was performed using the StatView packages.

Summary of results

Paper I

Small nerve fiber involvement in systemic lupus erythematosus

In this study we investigated fifteen consecutive and unselected patients with SLE and 15 age- and sex-matched controls to evaluate the IENF densities in skin punch biopsies from the distal part of the leg about 10 cm proximal to the lateral malleolus. The small-diameter nerve fibers were visualized using immunostaining against PGP 9.5. The IENF density was 8.0 $\pm$ 1.5/mm (range 5.0 - 9.9) in SLE as compared to 12.2 $\pm$ 3.8/mm (range 6.8 – 18.6) in healthy controls, P = 0.0006.

In conclusion, the IENF density was reduced in patients with SLE compared with age- and sex-matched controls.

Paper II

The effect of age and gender on epidermal nerve fiber density

This is the first study to establish normative values for IENF densities in healthy Norwegian subjects. One hundred and six healthy volunteers were recruited; mean age 49.0 $\pm$ 19.6 years (range 16.0 – 92.0). Two skin punch biopsies, 3 mm in diameter, were taken from the distal part of the leg about 10 cm proximal for the lateral malleolus and about one cm apart. The IENF were visualized using immunostaining against PGP 9.5. The mean IENF density was 12.4 $\pm$ 4.6 fibers/mm. Both increasing age and male gender were independently associated with decreasing IENF density. According to Bland and Altman plots (67) the mean difference in IENF densities by intraobserver analysis was 0.2 $\pm$ 1.2 fibers/mm, and the mean difference by interobserver analysis was 0.4 $\pm$ 1.5 fibers/mm, which were considered as acceptable in clinical practice.

Conclusion: The skin punch biopsy technique is easy to perform and the reliability of the method is good. Normal means and ranges for the IENF densities in a reference population have been established. The IENF densities are reduced with increasing age and also with male gender.

Paper III

Small-diameter nerve fiber neuropathy in systemic lupus erythematosus

In this study we investigated the involvement of small-diameter nerve fibers in 60 unselected patients fulfilling the revised ACR criteria for classification of SLE (28), 51 women and nine

men, mean age 43.2 ± 13.5 years.

The mean IENF density was 7.5 ± 3.8 fibers/mm, and the densities were below the lower reference limit in eight patients (13%). Six of these patients (10%) had normal NCS and had no PN according to the clinical judgement by the neurologist. These patients were thus classified as having a *pure* small-diameter nerve fiber neuropathy. Four patients (7%) were considered to have PN by the clinical judgement of the neurologist, and 13 patients (22%) had abnormal NCS.

The main conclusion from this study is that a certain proportion of patients with SLE, has an isolated small-diameter nerve fiber neuropathy, leaving larger nerve fibers unaffected.

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Paper IV

Intraepidermal nerve fiber densities in chronic inflammatory autoimmune diseases

This is the first study comparing small-diameter IENF densities in three different groups of patients with chronic inflammatory autoimmune diseases SLE, PSS, RA and healthy subjects. Sixty patients with SLE (mean age 43.2 ± 13.5 years), 61 patients with PSS (mean age 57.1 ± 14.6 years), and 52 patients with RA (mean age 57.4 ± 12.3 years) were examined. The IENF densities in skin punch biopsies from the distal leg about 10 cm proximal to the lateral malleolus were 7.5 ± 3.8 fibers/mm in SLE, 8.9 ± 4.0 in PSS, 10.9 ± 5.4 in RA and 12.4 ± 4.6 in healthy subjects. The densities were significantly less in SLE compared with RA and also with healthy subjects, and in PSS compared with healthy subjects.

These findings demonstrate that the involvement of small-diameter nerve fibers differs between different systemic autoimmune diseases probably reflecting differences in pathophysiology and organ affinity of the individual diseases.

Paper V

Peripheral neuropathy in primary Sjögren´s syndrome – a population based study

This study is the first to evaluate the prevalence of neuropathy including small-diameter nerve fiber involvement in an unselected cohort of patients with PSS applying the revised international criteria (46). Sixty-two unselected patients, mean age 57.1 ± 14.6 years were examined, 55 women and seven men. Seventeen patients (27%) were considered to have PN by an experienced neurologist. Thirty-four patients (55%) had abnormal NCS indicative of motor neuropathy in 19 patients (31%), sensory neuropathy in eight (13%), and sensorimotor neuropathy in seven patients (11%). Eight patients (13%) had concurrent PN at clinical examination and according to NCS. The mean IENF density in the leg was 9.2 ± 3.8

fibers/mm as compared with 9.6 $\pm$ 3.1 fibers/mm in the thigh, P = 0.37.

In conclusion, PN is a frequent finding in PSS. A majority of these patients with PN have findings indicative of a subclinical demyelinating motor neuropathy on NCS mainly reflected as increased F-wave latencies. Only two patients had IENF densities below lower reference limit with normal NCS and normal neurologic examination reflecting that a *pure* small-diameter nerve fiber neuropathy is infrequent in PSS.

General discussion

In paper I we showed that the densities of IENF in patients with SLE were reduced compared with age- and sex-matched controls and hypothesized that these findings may reflect the prevalent neuropathic symptoms and impaired warmth sense observed in these patients with otherwise normal NCS (68,69). The cohort was a non-selected small group of patients with SLE, and we revealed no association between IENF densities and variables like disease activity, disease duration, age, drug treatment or laboratory values. Only two patients had abnormal NCS, but the IENF densities in these two patients were not different from the others.

In paper II we investigated the IENF densities in healthy subjects to establish normative values from our own laboratory and define morphological criteria for small-diameter nerve fiber neuropathy. While others had failed to demonstrate an association between IENF densities and age and gender (12), we found that the IENF densities were reduced with increasing age and with male gender also. This is a logic finding as most neurologic and neurophysiological variables display an age-dependent association (70-72). The explanation for the lower IENF density in males is, however, more speculative. Females have greater sensitivity for small temperature changes and lower heat pain threshold compared with males in most parts of the body, and these modalities are conveyed through small-diameter nerve fibers (73). Other explanations like higher consumption of alcohol, exposure to more occupational chemicals, and more wear and tear with repetitive physical trauma amongst men are other possibilities.

To evaluate the intra- and interobserver variability of the method we constructed agreement plots (Altman plot) (67). In the medical literature the agreement between two methods is often expressed as correlation coefficients. Such use of correlation is, however, misleading because a high correlation coefficient does not imply that the two measurements agree. Correlation should be confined to test possible associations between two continuous variables. We found the intra- and interobserver variability of the method to be acceptable in clinical practice.

We revealed a large variability in IENF densities between different sections from the same punch biopsy. The intraobserver variability decreased in one study from 13.5 $\pm$ 12.6% to 6.4 $\pm$ 7.9% counting four sections as opposed to two sections (13). The variability of IENF densities based on counting of three sections from one punch biopsy in the distal part of the leg was 44.9 fibers^2/mm^2 in the study by McArthur et.al (12). The variability was even higher

in the thigh, 108.2 fibers^2/mm^2, and the physiological proximal-distal gradient reported by these authors could therefore be due to the methodology used with three sections counted in one biopsy only (12). In contrast to others we count at least three sections in each of two biopsies taken about one cm apart from the same anatomical area for IENF density estimation, and calculate the mean of IENF/mm from at least six sections. The variability in the distal part of the leg was in our study 21.2 fibers^2/mm^2 (not published). We therefore strongly recommend the use of two biopsies from the same anatomical area and to count at least three sections from each biopsy to reduce the variability of the method. We defined the morphological criterion for small-diameter nerve fiber neuropathy as below the mean IENF/mm - 1.96 SD (< 3.4 fibers/mm).

In paper III we confirmed the findings of reduced IENF densities in an unselected cohort of SLE patients compared with healthy subjects. In six (10%) patients the IENF densities were below the lower limit although both the NCS and clinical examinations were normal. In addition 11 patients (18%) had an abnormal NCS, which is in accordance with previous reports (34-37). However, only four patients were classified as having PN by the experienced. IENF densities below lower reference limit without abnormalities at NCS or at the clinical examination point to a selective process affecting small-diameter nerve fibers in some patients with SLE. This occurs in spite of low disease activity. Potential pathogenetic mechanisms could be immunoglobulin deposition on neural surfaces or a low-grade inflammation of small blood vessels with an activated endothelium and the triggering of apoptotic signals and subsequent degeneration of the small-diameter nerve fibers (74-76). These observations point to different pathogenetic mechanisms being operative in small- and large-diameter nerve fibers, respectively, and indicate that small-diameter nerve fiber involvement may be an early phenomenon in the disease development.

In paper IV we compared the IENF densities in three different patient groups, SLE, PSS, and RA. Although these diseases have several similarities, the pathogenesis and also clinical manifestations differ between the disease entities. We found that the IENF densities were significantly lower in patients with SLE compared with RA, but there were no significantly differences between SLE and PSS or PSS and RA. However, the IENF densities were significantly less in SLE and PSS compared with healthy subjects, but no such difference was observed between RA and healthy subjects. Although not significant, there were differences in IENF densities between the groups of patients (Figure 1), and with a larger number of patients in each group this differences may have become significant.

RA is characterized by predominantly mono-organ involvement, SLE with systemic or

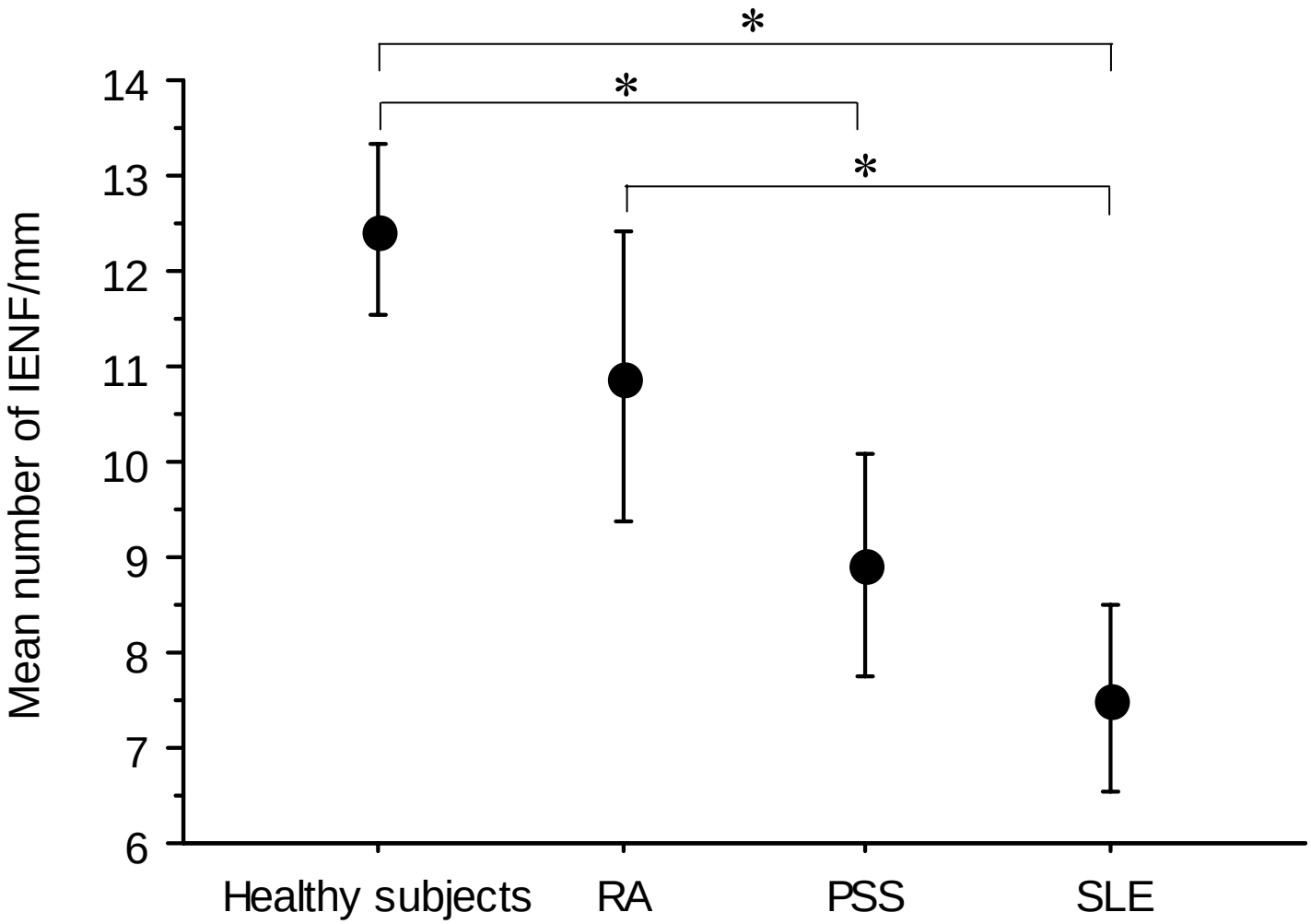
multi-organ involvement, and PSS somewhat in-between. In SLE the small-diameter nerve fibers may be a vulnerable target for a general immune activation, reflecting the systemic nature of the disease, or with a specific immune response targeting the nerve fibers. In patients with RA the small-diameter nerve fibers are not attacked due to the mainly joint involvement of the disease. In PSS, which has some characteristics of systemic as well as focal manifestations, the involvement of the small-diameter nerve fibers is somewhat in between the two other diseases.

In paper V we investigated the spectrum of PN including small-diameter nerve fiber neuropathy in an unselected cohort of patients fulfilling the new revised international diagnostic criteria for PSS (46). As far as we know this is the first study on neurologic features in a well-characterized group of unselected patients. We found an abnormal NCS in 34 patients (55%). This consisted of motor neuropathy in 19 patients (31%), sensory in eight patients (13 %), and sensorimotor in seven patients (11%). Fifteen out of the 19 patients classified as having motor neuropathy at NCS had an abnormal F-wave latency in $\geq$ 2 nerves as the only abnormality. The nerve conduction velocities between knee and ankle were significantly lower in patients with prolonged versus normal F-wave latency. Notably, the increased F-wave latencies were bilateral in all 15 patients, and for both groups of patients the conduction velocities were within the reference range. However, the more pronounced abnormalities in F-wave latency versus distal conduction velocity could theoretically be indicative of a more proximal than distal involvement, but none of the patients had a history, symptoms, or clinical findings of vertebral disc hernias. The F-wave latency is the single most sensitive neurophysiological parameter for detection of generalized motor nerve abnormalities (77,78). The long distance measured in the F-wave registration procedure improves the detection of minute abnormalities. In addition, the F-wave latency reference range is narrower than the conduction velocity reference range in the distal part of the nerve. In patients with SLE only four patients (7%) had reduced F-wave latencies indicating that the observations in patients with PSS are not accidental Although motor amplitudes were consistently lower in patients with increased F-wave latencies, these differences were nonsignificant, indicating that the prevailing neuropathic process is mainly demyelinating. However, the possibility of slight axonal involvement cannot be excluded. These observations contrast previous reports claiming that a symmetric sensorimotor PN followed by a symmetric pure sensory PN are most prevalent in patients with PSS (47,48). However, previous reports on this issue are hampered by selection bias due to changing diagnostic criteria and highly selected patient cohorts.

Seventeen patients (27%) achieved a diagnosis of neuropathy following clinical examination. The mean IENF density was 9.2/mm $\pm$ 3.8 in the leg as compared with 9.6/mm $\pm$ 3.1 in the thigh, P = 0.37, and the IENF densities in the leg were significantly lower than in healthy subjects. In contrast to patients with SLE, only two patients had IENF densities below normative values combined with normal NCS and clinical examination.

The prevalence of PN was 13% based on a combination of findings on clinical examination and NCS.

Figure 1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Mean number of IENF/mm
Healthy subjects
RA
PSS
SLE

Conclusions

By immunostaining the neuropeptide PGP 9.5 in punch skin biopsies, the small-diameter nerve fibers were visualized, quantitated, and normative values in a Norwegian population established. The intra- and interobserver variability were considered to be acceptable in clinical practice. Our studies demonstrate that the IENF densities are inversely associated with age, and men have lower densities than women. The variability of IENF densities between sections from the same biopsy is large, a finding with implications for the necessary numbers of sections counted and also for numbers of biopsies taken from each anatomical area.

Ten percent of the patients with SLE have a pure small-diameter nerve fiber neuropathy reflected by IENF densities below normative values, normal NCS, and normal neurological examination. Small-diameter nerve fiber loss is not associated with disease activity, autoantibodies, or medication, and the small-diameter nerve fibers are probably a target for the general immunological abnormalities taking place in these patients.

The IENF densities differ between autoimmune diseases like SLE, PSS, and RA. The densities are significantly less in patients with SLE compared to PSS and healthy subjects and in PSS compared to healthy subjects. Patients with RA have the same IENF densities as healthy subjects. These findings are indicative of different pathophysiology and organ affinity of the individual diseases.

Twenty-seven percent of the patients with PSS have PN as evaluated by an experienced neurologist. Fifty-five percent of patients with PSS have an abnormal NCS mainly reflecting motor neuropathy (31%) with increased F-wave latency, sensory neuropathy (13%), and sensorimotor neuropathy (11%). The group of PSS patients with abnormally increased F-wave latencies had significantly reduced conduction velocities compared with patients with normal F-wave latencies. The amplitudes were, however, not significantly reduced pointing to a demyelinating PN. Based on the combination of clinical examination and NCS the prevalence of PN was 13%. The IENF densities were reduced compared to healthy subjects on a group basis, but this was as part of a general neuropathic process affecting both small- and large-diameter nerve fibers. There were no gradient between IENF densities in the proximal thigh and distal leg probably reflecting normal IENF density distribution and not a general ganglionopathy in these patients.

The prevalence of small-diameter nerve fiber involvement differs between chronic systemic autoimmune diseases. In SLE the small-diameter nerve fibers are probably attacked early in the disease, which is reflected in a pure small-diameter nerve fiber neuropathy in

some of these patients. In PSS the small-diameter nerve fiber densities are reduced as part of a more general neuropathy, and in RA no significant reduction in small-diameter nerve fibers compared with healthy subjects could be detected. The pathogenesis is still largely unknown and should be explored in future studies.

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Table 1
Classification criteria for SLE (28)

1.	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
2.	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions.
3.	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient's history or physician's observation.
4.	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician.
5.	Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion.
6.	Serositis	a) Pleuritis – convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion or b) Pericarditis – documented by ECG or rub or evidence of pericardial effusion.
7.	Renal disorder	a) Persistent proteinuria greater than 0.5 g per day or greater than 3+ if quantitation not performed or b) Cellular casts – may be red cell, hemoglobin, granular, tubular or mixed.
8.	Neurologic disorder	a) Seizures – in the absence of offending drugs or known metabolic derangements; eg uremia, ketoacidosis, or electrolyte imbalance. or b) Psychosis – in the absence of offending drugs or known metabolic derangements; eg uremia, ketoacidosis, or electrolyte imbalance.
9.	Hematologic disorder	a) Hemolytic anemia – with reticulocytosis or b) Leukopenia – less than 4000/mm^3 total on two or more occasions or c) Lymphopenia – less than 1500/mm^3 on two or more occasions. or d) Thrombocytopenia – less than 100 000/ mm^3 in the absence of offending drugs.
10.	Immunologic disorder	a) Anti-DNA: antibody to native DNA in abnormal titer or b) Anti-SM: presence of antibody to Sm nuclear antigen or c) Positive finding of antiphospholipid antibodies based on: 1) An abnormal serum level of IgG or IgM anticardiolipin antibodies or 2) A positive test for lupus anticoagulant using a standard method or 3) A false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test.
11.	Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in

time and in the absence of drugs known to be associated with “drug induced lupus syndrome”.

Table 2
Revised international classification criteria for Sjögren´s syndrome (46)

I. Ocular symptoms: a positive response to at least one of the following questions:
   1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
   2. Do you have a recurrent sensation of sand or gravel in the eyes?
   3. Do you use tear substitutes more than 3 times a day?

II. Oral symptoms: a positive response to at least one of the following questions:
   1. Have you had a daily feeling of dry mouth for more than 3 months?
   2. Have you had recurrently or persistently swollen salivary glands as an adult?
   3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs – that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:
   1. Schirmer´s test, performed without anaesthesia ($\leq$ mm 5 in 5 minutes).
   2. Rose bengal score or other ocular dye score ($\geq$ 4 according to van Bijsterveld´s scoring system).

IV. Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score $\geq$ 1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue.

V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic test:
   1. Unstimulated whole salivary flow ($\leq$ 1.5 ml in 15 minutes).
   2. Parotid sialography showing the presence of diffuse sialectasiasis (punctate, caviatary or destructive pattern), without evidence of obstruction in the major ducts.
   3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion tracer.

VI. Autoantibodies: presence in the serum of the following autoantibodies:
   Antibodies to Ro (SSA) or La (SSB) antigens, or both.

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
   a) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive.
   b) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI).
   c) The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey.

Exclusion criteria

a) Past head and neck radiation treatment
b) Hepatitis C infection
c) Acquired immunodeficiency disease (AIDS)
d) Pre-existing lymphoma
e) Sarcoidois
f) Graft versus host disease
g) Use of anticholinergic drugs (since at time shorter than 4-fold the half life of the drug)

Errata

Paper V

Page 8, lines 5-7, should read: “Amplitudes of the motor responses tended to be lower in the group with abnormal F-wave latencies, but these differences did not reach statistical significance (Fig. 2B).”

Page 8, lines 12-14, should read: “The mean IENF densities in the leg were significantly lower in patients with PSS compared with normative values and as also observed in SLE patients.”